

GEOCHEMICAL SUPPORT FOR A CLIMBING HABIT WITHIN THE PALEOZOIC SEED FERN GENUS *MEDULLOSA*

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A long-standing problem in paleobotany is the accurate identification of the growth habits and statures of fossil plants. Tissue-specific analysis of stable carbon isotope ratios in plant fossils can provide an independent perspective on this issue. Lignin, a fundamental biopolymer providing structural support in plant tissues and the second most abundant organic material in plants, is ¹³C depleted by several parts per thousand, averaging 4.1‰, relative to other plant constructional materials (e.g. cellulose). With this isotopic difference, the biochemical structural composition of ancient plants (and inferred stature) can be interrogated using microscale in situ isotope analysis between different tissues in fossils. We applied this technique to a well-preserved specimen of the Late Paleozoic seed plant *Medullosa*, an extinct genus with a variety of growth habits that includes several enigmatic yet abundant small-stemmed species widely found in calcium carbonate concretions (“coal balls”) in the Pennsylvanian coal beds of Iowa, USA. It remains unclear which of the medullosans were freestanding, and recent analysis of the medullosan vascular system has shown that this system provided little structural support to the whole plant. The leading hypothesis for small-stemmed medullosan specimens predicts that cortical tissues could have provided additional structural support, but only if they were lignified. The expected isotopic difference between lignified tissue and unlignified tissue is smaller than that expected from pure extracts, for the simple reason that even woody tissues maximally contain 40% lignin (by mass). This reduces the expected maximum difference between weakly and heavily lignified tissues by 60%, down to ~0.5‰–2‰. Analysis of the medullosan stem reveals a consistent difference in isotope ratios of 0.7‰–1.0‰ between lignified xylem and cortical tissues. This implies low abundances of lignin (between 0% and 11%) within the cortex. This inferred structural biochemistry supports hypotheses that the peripheral portions of these medullosan stems were not biomechanically reinforced to permit the plants to grow as freestanding, arborescent trees. A number of climbing or scandent medullosans have been identified in the fossil record, and this mode of growth has been suggested to be common within the group on the basis of observations from comparative biomechanics, hydraulics, and development. Finally, this mode of growth is common in several clades of stem group seed plants, including *Lyginopteris* and *Callistophyton*, along with *Medullosa*. This study provides further support for ideas that place a great portion of early seed plant diversity under the canopy, rather than forming it.

Keywords: paleoecology, carbon isotopes, paleobotany, lignin, Carboniferous, coal.

Introduction

A central problem in paleobotany is determining the architecture of fossil plants. This is complicated by two primary factors: first, fossil plants are commonly preserved as disarticulated organs, and second, physical damage incurred during fossilization can obscure the original morphology. This problem is particularly acute for two types of plants, narrow-stemmed plants and specimens preserved as compression fossils. It can be difficult to determine whether these plants were freestanding or climbed on existing supports (Niklas 1978, 1992; Mosbrugger 1990). In this article, we address the question of whether a particular morphology of the Paleozoic seed plant genus *Medullosa* had a climbing or a freestanding architecture (fig. 1). We focus on this particular growth form

because previous work has suggested that the peripheral tissues of the plant were instrumental in providing structural support for its large petioles, which reached up to 9 m in length (Mosbrugger 1990); we examine this hypothesis, using carbon isotope analysis of fossil material.

Several approaches have been explored to constrain fossil plant stature. These include developing analogies to living plants (Pfefferkorn et al. 2001), biophysical modeling (Niklas 1992), physical analysis of fossil plant cells (Rowe et al. 1993), and hydraulic modeling (Rowe and Speck 2004; Wilson et al. 2008). However, each of these methods suffers limitations: analogies can be imperfect or misleading, biophysical models require assumptions about tissue strength, and hydraulic models are built using isolated organs. Chemical analysis of fossil tissue provides an independent perspective from these methods by offering insight into the biochemical construction of fossil plant tissues. Previous work has shown that stable isotope ratios of biochemical compounds that confer structural support in plants are robust to diagenetic

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change, allowing the construction of fossil plant tissues to be directly observed from fossils themselves (Boyce et al. 2003).

Structural properties of plant tissues are a consequence of cell wall biochemistry. The presence of lignin, a complex macromolecular biopolymer derived from modified phenylalanine monomers, is a fundamental component of plant structural integrity (Taiz and Zeiger 2002). Lignin provides resistance to bending and tensile forces in cells, providing stability to the water transport system in wood and also permitting cells to resist bending forces, including gravity and wind loading (Tyree and Ewers 1991; Tyree and Zimmermann 2002; Rogers and Campbell 2004; Bonawitz and Chapple 2010; Spicer and Groover 2010). Highly lignified, narrow cells allow trees to reach heights over 100 m (Tyree and Ewers 1991; Tyree and Zimmermann 2002; Sperry 2003). In arborescent angiosperms, nonconductive fiber cells fill this role; they are produced from the vascular cambium along with vessel elements, which form conduits for water transport (Sperry et al. 2007). In conifers, tracheids are highly lignified and narrow (<40 μm in diameter) and serve to transport water while providing structural support at the same time; they fulfill this dual role by employing highly porous membranes between adjacent cells (Pittermann et al. 2005, 2006). Because of its hydrophobic and mechanical properties, lignin is necessary for and essential to the function of vascular plants (Tyree and Zimmermann 2002; Edwards 2003; Sperry 2003; Baas et al. 2004; Brodribb 2009; Spicer and Groover 2010). Plants with reduced cell wall lignin contents exhibit malfunctioning water transport tissue and experience a number of deleterious physiological effects (e.g., increased wall failure and cavitation, reduced stomatal conductance, and carbon assimilation up to mortality shortly after germination; Coleman et al. 2008; Wagner et al. 2009; Bonawitz and Chapple 2010). These numerous lethal effects illustrate the central adaptive role of lignified tissue in the evolution of vascular plants (Freidman and Cook 2000).

Knowledge of the distribution of lignin in fossil plants is critical to understanding the biomechanical properties of plant tissues and thereby important for informing hypotheses about the life habits and modes of growth of these plants. Spectroscopic methods have been used to identify lignin in fossil plant tissues; these methods rely on correlating spatially discrete differences in cell wall chemistry (e.g., aromatic carbon moieties) with cell wall layering and morphology (Boyce et al. 2002, 2003, 2010). This approach is rapid and nondestructive and has the potential to inform subcellular-level distributions of fossil plant biopolymers, but complications can arise because aromatization of aliphatic plant compounds provides an additional source of aromatic products. Careful experiments can deconvolve these signals, and isotopic methods can help calibrate their results. Although isotopic methods are (minimally) destructive, they remain attractive because of their robustness with regard to diagenetic change.

Carbon Isotope Ratios of Fossil Plant Tissues Record Biophysical Information

The carbon isotope composition of plant tissues is a result of many factors (e.g., autotrophic metabolism, carbon-

concentrating mechanisms, water status, climate). In isolation, values of carbon isotope ratios of single tissues are underconstrained and shed little light on the biochemistry and biophysics of fossil plants. However, measuring and differentiating carbon isotope ratios from different tissues within the same fossil provides a metric that can be translated directly into biopolymer abundances (e.g., lignin content). Previous work focused on differentiating lignified tissue from cellulose-rich tissue in some of the earliest land plant fossils (e.g., Boyce et al. 2003). Here we take a different approach: we compare the isotope ratios of putative structural tissues to those that we independently know were lignified. By differing their isotopic compositions, we can ascertain whether additional tissue types were lignified and may have therefore provided additional structural support for the plant.

Isotopic differences between plant tissues are a consequence of structural biochemistry. Across a wide diversity of extant clades, the carbon isotope ratios of lignin are systematically lower than bulk plant biomass and other structural biopolymers, primarily cellulose (Benner et al. 1987; Loader et al. 2003; Hobbie and Werner 2004; Eglin et al. 2008). Despite this clear and persistent pattern, the cause remains obscure, even with considerable progress unraveling the lignin biosynthetic pathway (Boudet 1998; Humphreys and Chapple 2002; Rogers and Campbell 2004; Spicer and Groover 2010).

There are two hypotheses that explain the ^{13}C depletion widely found in lignin. The first suggests that lignin is ^{13}C depleted relative to cellulose because of systematic differences in the isotopic composition of amino acids (Benner et al. 1987; Hobbie and Werner 2004). Lignin monomers are derived from the amino acid phenylalanine, which in turn is derived from triose phosphate via the shikimic acid pathway (fig. 2; Humphreys and Chapple 2002; Boerjan et al. 2003; Vanholme et al. 2010). The isotopic composition of individual amino acids can vary as a function of metabolism in both sign and magnitude (Abelson and Hoering 1961; Fogel and Tuross 1999; Hayes 2001; Scott et al. 2006). Where these differences have been studied in plants, phenylalanine has approximately the same isotopic composition as bulk biomass, within 2‰ (Winters 1971; Fogel and Tuross 1999). These differences are probably too small to account for the systematic isotopic difference observed between lignin and cellulose. An additional important observation suggests an alternative hypothesis. By examining the magnitude of isotopic difference within a single plant, it has been observed that as lignin content increases in a single tissue, the magnitude of the difference between cellulose and lignin decreases (from 7‰ in lightly lignified tissues to as little as 3‰ in woody tissues; Hobbie and Werner 2004). These observations can be explained by a kinetic fractionation and distillation of a finite monomer reservoir in the early steps of the transformation from phenylalanine to lignin monomers, rather than underlying differences in amino acid $\delta^{13}\text{C}$. In this second hypothesis, a strong kinetic isotope effect on the alpha carbon of phenylalanine would be imparted during deamination by phenylalanine ammonia lyase (PAL; fig. 2), the first irreversible step in phenylprenoid biosynthesis. This mechanism can, in principle, explain the negative $\delta^{13}\text{C}$ composition of both lignin monomers (Hobbie and Werner 2004) and degradation products (Tenailleau et al. 2004).



Fig. 1 Three different growth habits of medullosans. **A**, Reconstruction of a self-supporting medullosan based on material from the Bernice Basin, Pennsylvania (Pfefferkorn et al. 1984; Wnuk and Pfefferkorn 1984). Compound leaves are simplified to permit the stem to be seen. Plant is ~ 4 m tall. **B**, Reconstruction of lax-stemmed *Medullosa thompsoni* from Iowa (Andrews 1940). Leaves are drawn as drooping to fit within the frame; in the in-life position, petioles would probably be $\sim 60^\circ$ from horizontal (Pfefferkorn et al. 1984). Plant is ~ 4 m tall. **C**, Permineralized Permian medullosan from Chemnitz, Germany (Sterzel 1918). Stem is ~ 20 cm in diameter. Note the erect stature.

Two observations suggest that the systematic isotopic difference between $\delta^{13}\text{C}$ of cellulose and that of lignin has been conserved over geological time. First, as described above, analysis of lignin and cellulose extracted from the same plant across a wide range of terrestrial plant clades (e.g., conifers, angiosperms), ecological niches (e.g., trees, shrubs, seagrasses), and organs (e.g., leaves, wood) has shown that the difference between the carbon isotope ratios of these compounds is always of the same sign and similar magnitude (commonly 2‰ – 7‰ but averaging $4.1\text{‰} \pm 0.6\text{‰}$; Benner et al. 1987; Hobbie and Werner 2004). Second, the core sequence of reactions within the lignin biosynthetic pathway in lycophytes, ferns, conifers, and angiosperms appears to be conserved, suggesting that key enzymes at the core of the phenylpropanoid pathway have changed little over the past 400 million years (Boudet 1998; Humphreys and Chapple 2002; Boerjan et al. 2003; Hobbie and Werner 2004; Rogers and Campbell 2004; Weng et al. 2008; Vanholme et al. 2010). This pattern implies that we can reasonably expect similar isotopic chemistry in ancient lineages of seed plants.

Finally, it is important to consider whether the difference between $\delta^{13}\text{C}$ compositions of lignin and of cellulose would be preserved in the fossil record. Previous work has shown that differences can be observed, even in the fossils of some of the earliest land plants (Boyce et al. 2003). Recent work on stepwise oxidation of functional groups within plant biopolymers has demonstrated that the anomalously ^{13}C -depleted carbon pool resides within the phenolic group of lignin monomers rather than the more labile methoxyl groups (Greule et al. 2009; fig. 2). Site-specific carbon isotope analysis of the lignin derivative vanillin corroborates this observation:

carbon molecules within the phenolic group are ^{13}C depleted, whereas methoxyl carbons are ^{13}C enriched (Tenailleau et al. 2004). With the source of the $\delta^{13}\text{C}$ signal located within the stable backbone of the lignin monomer, these differences have the opportunity to be preserved in plant fossils.

Lignin is among the most degradation-resistant components of plants. Covalent linkages of lignin monomers provide resistance to physical degradation (Boerjan et al. 2003), and there are few enzymatic pathways that degrade lignin (Kirk and Farrell 1987; Hirai et al. 1994). Lignin is commonly consumed by black and white rot fungi using an oxygen-dependent pathway, and anaerobic remineralization of lignin is currently unknown. Even if such a pathway exists, it should proceed at a far slower rate than aerobic pathways, increasing the possibility of fossilization and preservation of lignin in deep time. Analysis of the early taphonomic stages of plant decay shows that lignin abundances change very little in aquatic environments (Hernes et al. 2001). In deep time, empirical morphological, ultrastructural, and pyrolytic evidence suggests that lignin is preserved, even in fossils of Late Silurian age (Niklas and Pratt 1980; Friedman and Cook 2000; Edwards 2003; Taylor and Wellman 2009).

And finally, even if it is likely to be preserved, it is important to ask whether this carbon isotope difference is analytically resolvable in fossil plants. A complication arises because plant cell walls are intimate mixtures of structural macromolecules, primarily cellulose, lignin, and hemicellulose (Zugenmaier 2008). To express this relationship graphically, we collected measurements of lignin content in various plant tissues from the literature, including bulk measurements, tissue-specific measurements, and measurements in crop

plants and low-lignin mutants (Harlow 1970; Lechtenberg et al. 1974; Jung and Vogel 1986; Liese 1992; Moore and Jung 2001). Much of these data come from agricultural feed literature; low lignin contents are more energy efficient for ruminants to digest (Lechtenberg et al. 1974; Jung and Vogel 1986). Low-lignin mutants of domesticated angiosperms have also attracted wide interest as a potential source of cellulose for biofuel applications. The biochemical properties of lignin that make it useful for structural support and water transport (hydrophobicity, strong cross-linkages between monomers, and tight bonding with cellulose) also make it energetically expensive to remove from plant cells in order to concentrate cellulose and polysaccharides. Research has focused on low-lignin mutants of *Zea mays* (corn) and *Triticum*

aestivum (wheat), which often grow as dwarf or small-statured plants because of their reduced capacity for photosynthesis and structural support. As shown in figure 3, even in low-lignin mutants, lignin still makes up more than 5% of bulk plant tissues, illustrating its critical role in plant physiology, biomechanics, and defense.

Expected Difference between $\delta^{13}\text{C}$ of Lignified Tissue and That of Cellulose-Rich Tissue

We plotted the expected value of the difference between lignified tissue and cellulose-rich tissue ($\Delta\delta^{13}\text{C}_{\text{expected}}$) as the product of lignin percentage (F_{lignin}) and the difference between the carbon stable isotope composition of cellulose

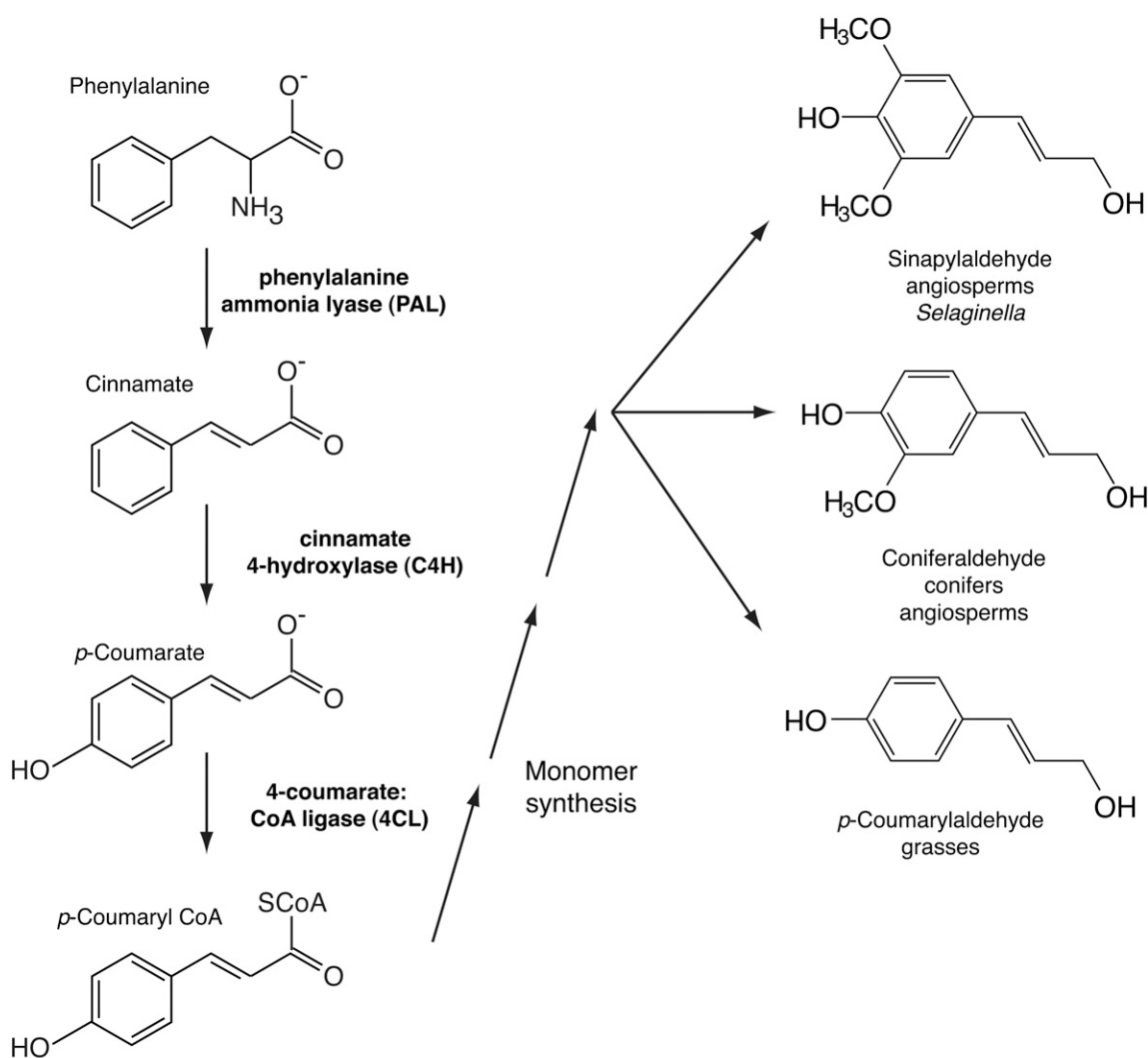


Fig. 2 Simplified diagram of the lignin biosynthetic pathway showing lignin monomers (right) and critical early reactions (bold), modified from Bonawitz and Chapple (2010). A possible source of the ^{13}C -depleted isotopic signal found in lignin is a kinetic isotope effect on the α carbon of phenylalanine during deamination by the enzyme phenylalanine ammonia lyase (PAL; Hobbie and Werner 2004). This is the first irreversible step in phenylpropanoid biosynthesis. Recent work suggests that the methoxyl (CH_3O) groups of sinapylaldehyde and coniferylaldehyde have $\delta^{13}\text{C}$ compositions indistinguishable from bulk lignin values (Greule et al. 2009). This indicates that the isotopic signal is held by the backbone of the molecule and is preservable in geological samples despite the expected loss of methoxyl groups during diagenesis (Hedges et al. 1985; Spiker and Hatcher 1987; Hatcher and Lerch 1991).

and lignin ($\delta^{13}\text{C}_{\text{cellulose}} - \delta^{13}\text{C}_{\text{lignin}}$). This relationship is expressed mathematically in equation (1):

$$\Delta\delta^{13}\text{C}_{\text{expected}} = (\delta^{13}\text{C}_{\text{cellulose}} - \delta^{13}\text{C}_{\text{lignin}}) \times F_{\text{lignin}}. \quad (1)$$

Lignin fractions vary from 0% to ~40%; even that of woody tissues does not exceed 45% (table 1). Figure 3 shows the range of expected differences in carbon stable isotope values for a variety of tissues. The slope of the function changes on the basis of the magnitude of the $\delta^{13}\text{C}$ difference between lignin and cellulose, but the measurable portion of this difference decreases with decreasing lignin amount, to less than 2‰. Two expected relationships can be expressed here. The first considers the isotopic fractionation to be linear and independent of lignin concentration, with an average slope of $4.1\text{‰} \pm 0.6\text{‰}$ (eq. [1]; fig. 3, solid line and gray triangle). The second is a polynomial based on empirical evidence that the isotopic fractionation between lignin and cellulose decreases with increased lignin content (Hobbie and Werner 2004) be-

cause of isotope mass balance constraints in the lignin biosynthetic pathway. Both relationships, however, yield similar isotopic predictions (ranging from 0.2‰ to 1.5‰) in the range of lignin abundances in plant tissues. At lignin abundances of less than 22%, the linear model provides a lower estimate of the isotopic difference; the opposite is true for lignin abundances greater than 22%. That the isotopic difference is non-zero across all tissue types shows that lignin abundances can be resolved with tissue-specific isotope ratio analyses.

Given the low expected isotopic difference between tissues of varying lignification, our analytical approach is constrained by instrument limitations. If we require precise and accurate measurements on the order of 0.25‰, we are limited to microsampling (e.g., millimeter-size microrotary drilling) methods using elemental analysis isotope ratio mass spectrometry. In situ measurements using secondary ion mass spectrometry offer substantially better spatial resolution (spot sizes between 5 and 25 μm) and integrate over much smaller sample volumes, but they flirt with the level of isoto-

Table 1

Percent Abundance of Lignin in Tissues and Cells from Gymnosperms and Angiosperms

Species	Identity	Tissue	Lignin (%)	Reference
<i>Abies balsamea</i>	Conifer	Wood	29	Harlow 1970
<i>Picea glauca</i>	Conifer	Wood	27	Harlow 1970
<i>Pinus strobus</i>	Conifer	Wood	29	Harlow 1970
<i>Tsuga canadensis</i>	Conifer	Wood	33	Harlow 1970
<i>Thuja occidentalis</i>	Conifer	Wood	31	Harlow 1970
<i>Acer rubrum</i>	C ₃ angiosperm	Wood	24	Harlow 1970
<i>Betula papyrifera</i>	C ₃ angiosperm	Wood	19	Harlow 1970
<i>Fagus grandifolia</i>	C ₃ angiosperm	Wood	22	Harlow 1970
<i>Populus tremuloides</i>	C ₃ angiosperm	Wood	21	Harlow 1970
<i>Ulmus americana</i>	C ₃ angiosperm	Wood	24	Harlow 1970
<i>Zea mays</i> :				
Wild type	C ₄ angiosperm	Stalk	9.3	Lechtenberg et al. 1974
bm3 mutant	C ₄ angiosperm	Stalk	5.5	Lechtenberg et al. 1974
<i>Dactylis glomerata</i>	C ₃ grass	Bulk	6.6–8.1	Jung and Vogel 1986
<i>Panicum virgatum</i>	C ₄ grass	Bulk	3.5–8.1	Jung and Vogel 1986
Unidentified	Legume	Fiber cells	5	Moore and Jung 2001
Unidentified	Legume	Fiber cells	17	Moore and Jung 2001
Unidentified	Legume	Fiber cells	18	Moore and Jung 2001
Unidentified	Legume	Fiber cells	20.5	Moore and Jung 2001
Unidentified	Legume	Fiber cells	24	Moore and Jung 2001
Unidentified	Legume	Fiber cells	27	Moore and Jung 2001
Unidentified	Legume	Fiber cells	27	Moore and Jung 2001
Unidentified	Legume	Fiber cells	27	Moore and Jung 2001
Unidentified	Legume	Fiber cells	31	Moore and Jung 2001
Unidentified	Legume	Fiber cells	40	Moore and Jung 2001
Unidentified	Legume	Fiber cells	33	Moore and Jung 2001
Unidentified	Grass	Fiber cells	3	Moore and Jung 2001
Unidentified	Grass	Fiber cells	4	Moore and Jung 2001
Unidentified	Grass	Fiber cells	4.2	Moore and Jung 2001
Unidentified	Grass	Fiber cells	4.5	Moore and Jung 2001
Unidentified	Grass	Fiber cells	4.5	Moore and Jung 2001
Unidentified	Grass	Fiber cells	8.5	Moore and Jung 2001
Unidentified	Grass	Fiber cells	12	Moore and Jung 2001
Unidentified	Grass	Fiber cells	12.5	Moore and Jung 2001
Unidentified	Grass	Fiber cells	13	Moore and Jung 2001
Unidentified	Grass	Fiber cells	12.5	Moore and Jung 2001
Unidentified	Grass	Fiber cells	15	Moore and Jung 2001

Note. Angiosperm wood averages 22% lignin; gymnosperm wood averages 29% lignin.

pic precision required to provide robust estimates of lignin abundances. This limits the types of questions that can be answered with $\delta^{13}\text{C}$ analysis: for example, the degree of lignification of the primary cell wall is an important biochemical control over plant hydraulics (Boyce et al. 2004), but these differences in lignin composition occur over an anatomical feature less than $3\ \mu\text{m}$ wide in cross-section. This is too small to resolve with the instrument resolution that is currently available. However, the spatial resolution of microdrilling will permit us to ask questions about biomechanics, if not hydraulics. To map the biophysical properties of permineralized fossil plant stems, microdrilling spatial resolution is adequate to provide multiple tissue-specific samples of a given fossil plant.

The Late Paleozoic Seed Plant *Medullosa*

We chose to study this problem using a seed plant with disputed architecture and habit, a specimen of the stem group seed plant *Medullosa*. This enigmatic plant is a good study candidate for two reasons. First, as one of the earliest seed plants, *Medullosa* is placed at a key evolutionary junction. Medullosans are thought to be a sister group to the clade that includes cycads, gnetales, conifers, and angiosperms (Doyle 2006; Hilton and Bateman 2006). Second, other features of its physiology, particularly its highly conductive xylem (Wilson et al. 2008; Wilson and Knoll 2010) and

spacing between stomata and veins (Boyce et al. 2009), are physiologically similar to those in early angiosperms but were constructed with different anatomical features and developmental mechanisms. Investigation of the physiological capabilities of *Medullosa* gives a comparative example for early angiosperms, which have been hypothesized to have crossed several functional barriers to achieve ecological dominance (Sperry 2003; Hacke et al. 2006; Sperry et al. 2007).

The genus *Medullosa* is known to contain a diversity of architectural forms and is probably equivalent to a modern order of plants (Andrews and Mamay 1953; Delevoryas 1955; Basinger et al. 1974; Pfefferkorn et al. 1984; Wnuk and Pfefferkorn 1984; Hamer and Rothwell 1988; Dunn et al. 2003; Dunn 2006; Taylor et al. 2008). This architectural diversity shares several interesting anatomical features: anastomosing xylem segments, compound petioles up to 9 m in length, adventitious roots (as opposed to taproots), and large leaf bases (Delevoryas 1955; Taylor et al. 2008).

On the basis of their size (and, in one case, on the basis of the preservation of three-dimensional branching; Sterzel 1918; fig. 1C), some medullosan seed ferns were freestanding (fig. 1A). There are also medullosans that undoubtedly were climbers or were scandent, on the basis of the growth form of their branch tips and the discovery of structures on their leaves that appear to be specialized for climbing (Hamer and Rothwell 1988; Krings and Kerp 2006). However, there are

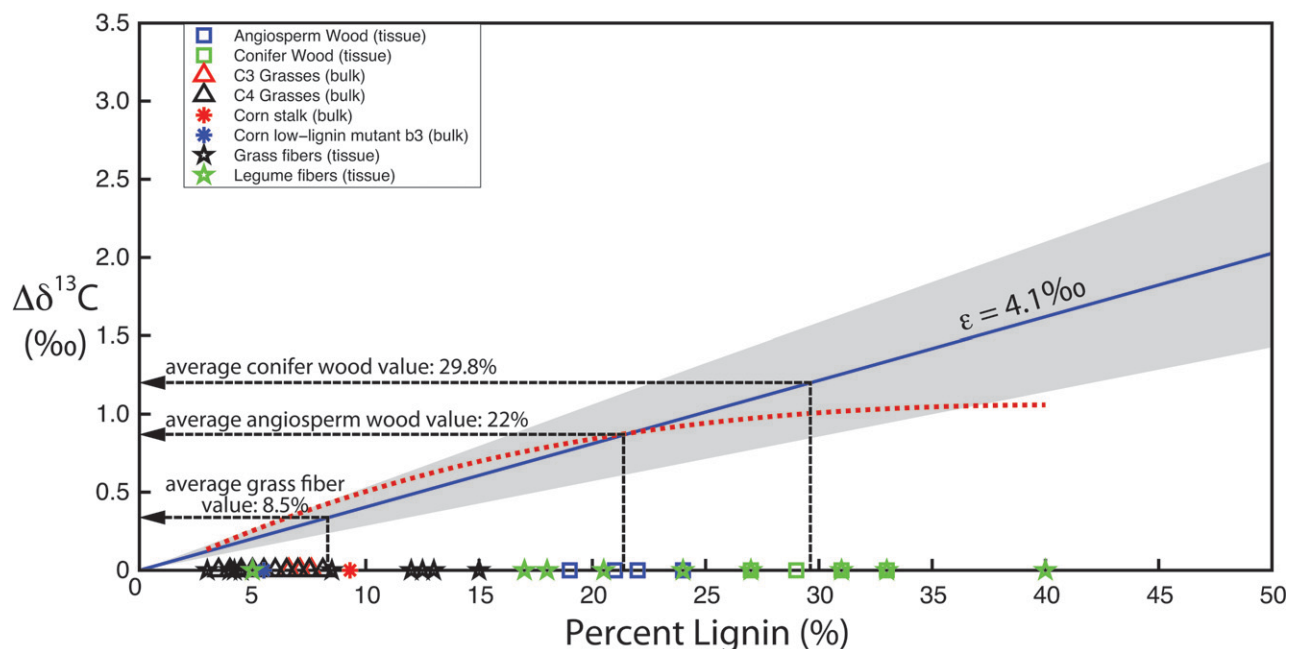


Fig. 3 Values of the isotope ratio difference between plant tissues as a function of lignin composition on the basis of a model of the fractionation between lignin and cellulose within a plant. A linear model of the isotope ratio difference between plant tissues is shown (solid line; gray area is 2σ) alongside a polynomial model (red dashed line; $y = -0.0007x^2 + 0.0536x + 0.0078$; derived from Hobbie and Werner 2004). Given the lignin composition of different tissues, the Y-axis shows the expected observable range of differences in fossils. Woody tissues are expected to be isotopically lighter than nonwoody tissues, but note that for many tissues, the observable range is less than 2‰ . There is a strong similarity between the linear and the polynomial models in the region of lignin abundances expected for plant tissues, but note that the polynomial model slightly underestimates lignin concentration in lightly lignified tissue and slightly underestimates the fractionation in lignin-rich cells (e.g., fibers).

many medullosan seed ferns that have small stems and large leaf areas but lack anatomical features that help distinguish whole-plant morphology, such as climbing hooks on fronds or thick stilt roots to enhance a freestanding position, as is found in extant mangroves. Reconstructions have shown these plants to be either freestanding (fig. 1A) or wilting, with a lax morphology (fig. 1B). A number of detailed studies have examined growth form and variation in the genus *Medullosa* (Pfefferkorn et al. 1984; Wnuk and Pfefferkorn 1984; Mosbrugger 1990). Specifically, Mosbrugger (1990) hypothesized that for small-diameter medullosans, the cortical tissue must have been the main source of support, but only if it was composed of lignified cells.

Methods

This study captures an opportunity to perform chemical analysis on a fossil plant with a well-preserved internal anatomy. The specimen analyzed here is a medullosan stem that was permineralized within a Carboniferous Period (Pennsylvanian) coal ball from the Shuler Mine near Des Moines, Iowa (41°38'9.63"N, 93°51'12.84"W). Two specific ana-

tomical features, the amount of parenchyma within the primary body and the lack of strand-type cortical development, suggest that this is a specimen of *Medullosa anglica* var. *ioensis* (Andrews and Kernen 1946). Several coal balls containing petioles of the genus *Myeloxylon*, also from the Shuler Mine, were prepared for comparative study. All specimens come from the Harvard University Paleobotanical Collections but lack specimen numbers.

Coal balls were polished on a vibrolap, using alumina grit and water. Sample powders were microdrilled, using a fine rotary drill press. Samples were extracted from the best-preserved tissues: secondary xylem and cortical tissues found within the stem (fig. 4). Primary xylem was not clearly preserved and therefore was not sampled, but early-developing secondary xylem is indicated in figure 5. Given the heterogeneous nature of medullosan cortical tissue (termed dictyoxylon cortex, a common tissue type in Paleozoic pteridosperms), we sampled small patches of dark cells that are commonly interpreted as fibers ("fiber-rich" samples) and zones within the dictyoxylon-type cortical tissue that contained fewer fibers ("mixed samples"; fig. 5). In addition to fiber-rich and mixed samples from the medullosan stem, fiber-rich and mixed tissues were sampled from medullosan petioles, along with coal

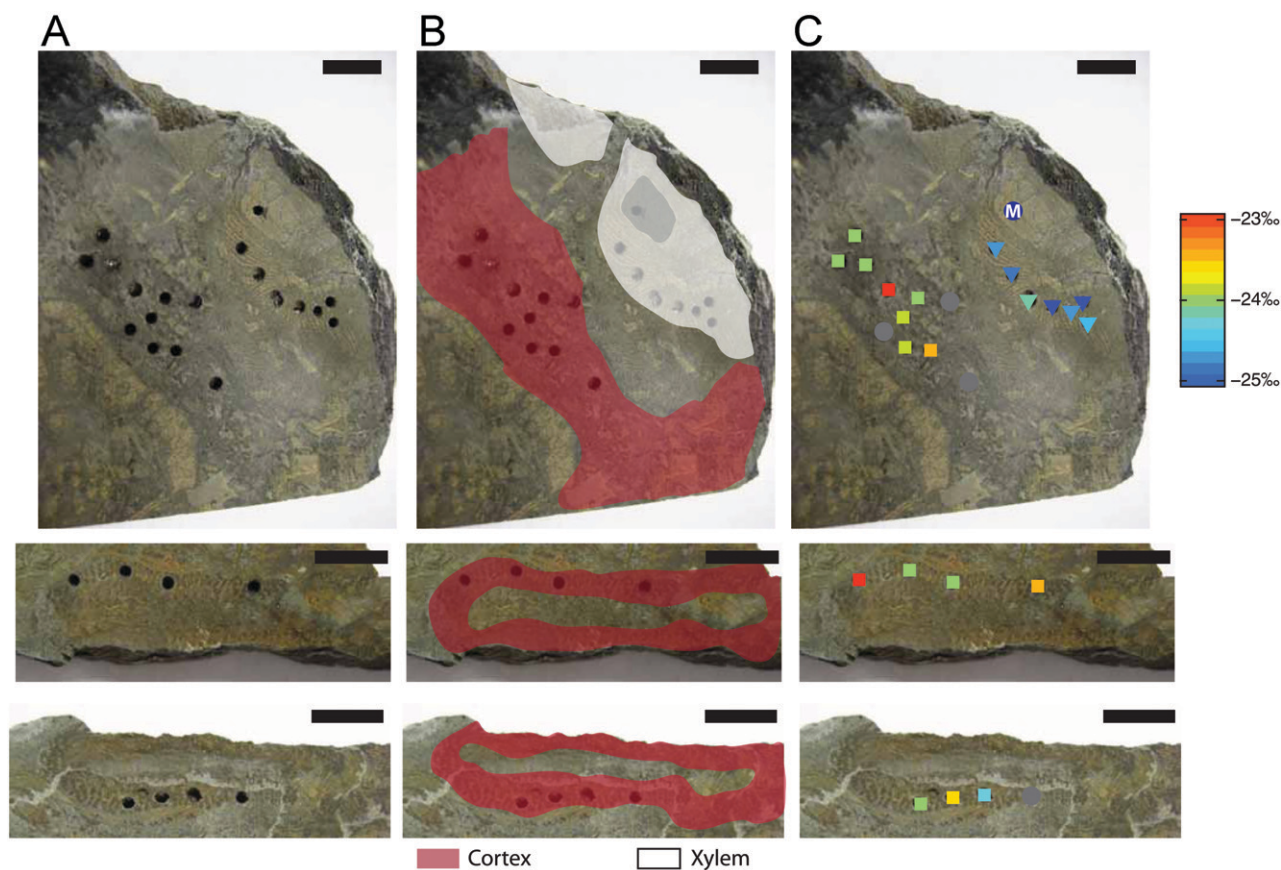


Fig. 4 Medullosan fossils, anatomical interpretation, and spatially resolved $\delta^{13}\text{C}$ data. A, Unprocessed images of fossils. B, Overlays of anatomical interpretation: red areas are zones of cortical tissue, white areas are xylem (xylem strands are not resolvable in petioles and are not annotated), gray shading is matrix. C, $\delta^{13}\text{C}$ values overlaid on fossils. Hotter colors indicate areas that are more ^{13}C enriched; gray shading is used for samples with too little mass to obtain accurate $\delta^{13}\text{C}$ data, M denotes matrix sample. Top panels are results for a medullosan stem; bottom panels are images from petioles. Note that cortical tissues (including petiole cortical tissue) are consistently more ^{13}C enriched than xylem.

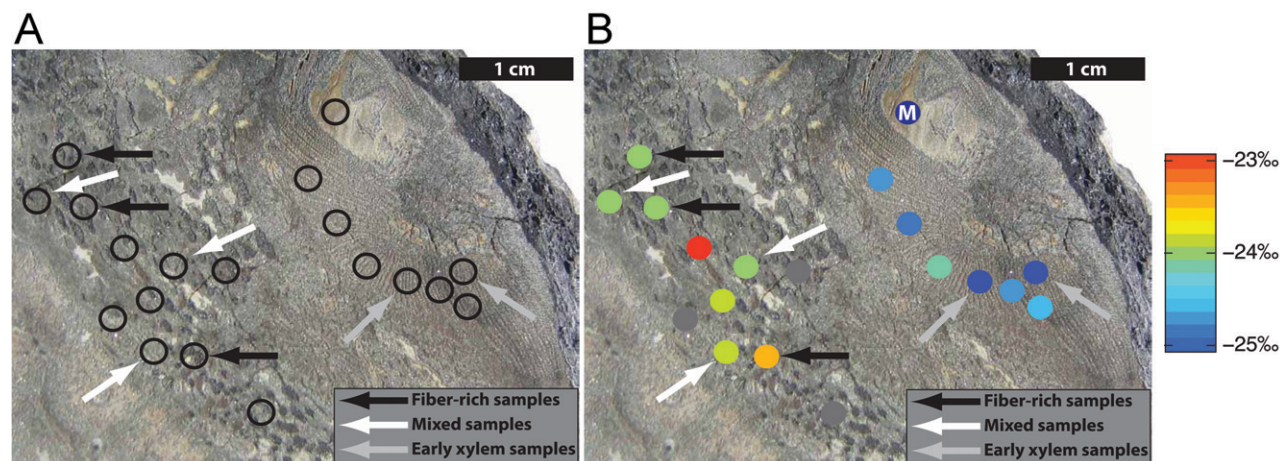


Fig. 5 A detailed look at cortical and xylem samples. *A*, Sample locations are indicated by black circles. Samples that contained mostly dark cortical cells (“fibers”) are denoted by black arrows, samples that contained fewer fiber cells (“mixed samples”) are denoted by white arrows, and secondary xylem produced early in the plant’s life cycle (“early xylem samples”) are denoted by gray arrows. *B*, Isotopic compositions of all samples. Note that there is no significant difference between fiber-rich and mixed samples and that the $\delta^{13}\text{C}$ values are maximally different between early xylem samples and all cortical samples. This discrepancy suggests that the systematic offset between cortex and xylem is a function of biochemistry rather than tissue age.

ball matrix. Comparing these two types of samples within the stem and between the stem and the petioles can help to identify whether fibers within the dictyoxylon cortex were lignified. Samples required ~ 10 mg of coal ball material to yield sufficient gas for mass spectrometry (~ 100 μg of CO_2). This limits our ability to analyze fibers in isolation.

Powders were measured in tin combustion boats and decarbonated using HCl chemical vapor fumigation. Carbon stable isotope ratios were measured at the University of California, Davis, Department of Biology Stable Isotope Laboratory, using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 continuous flow isotope ratio mass spectrometer. Samples were combusted at 1020°C in a reactor packed with chromium oxide and silvered cobaltous/cobaltic oxide. Following combustion, oxides were removed in a reduction reactor (reduced copper at 650°C), and the helium carrier flowed through a water trap (magnesium perchlorate). Sample analyses were interspersed with several replicates of two different laboratory standards calibrated against National Institute of Standards and Technology standard reference materials (IAEA-CH7 and NBS-22). Analytical uncertainty was less than 0.06‰ . Data are reported in delta notation relative to a PDB standard and were analyzed using MATLAB and coregistered with specimen images using Adobe Illustrator.

Results

In the permineralized medullosan stem, vascular tissue (interpreted to be lignified) is consistently and systematically ^{13}C depleted with respect to cortical tissue. Furthermore, xylem is consistently depleted from fiber-rich and mixed samples from the cortex; the fibers have an isotopic composition that is not significantly different from other parts of the cortical tissue. Early-developing secondary xylem tissue is maxi-

mally different from cortex samples, which are ^{13}C enriched by 1.04‰ . Samples of cortical tissue taken from petioles are slightly more enriched in ^{13}C relative to stem cortex, but the magnitude of the offset is approximately 0.4‰ . $\delta^{13}\text{C}$ standard deviation within tissues is somewhat high but is distinct between tissues: xylem averages $-24.72\text{‰} \pm 0.41\text{‰}$, whereas cortex averages $-23.67\text{‰} \pm 0.38\text{‰}$.

The 1.04‰ offset between xylem and cortex implies that lesser amounts of lignin were present in the cortical tissues. With an estimated isotopic fractionation of 4.1‰ , the average offset between our tissues (fig. 6) predicts a difference in lignin content between xylem and cortex of $18\% \pm 2\%$. If medullosan vascular tissue contained a lignin abundance similar to that found in angiosperm wood, then it would yield approximately 3%–6% lignin in the cortex; if medullosan wood contained a lignin abundance that was more similar to that found in conifer wood, then we would predict approximately 7%–11% lignin in the cortex. These values are most similar to those for hemp fibers and wheat straw, respectively (Zugenmaier 2008).

These estimates of lignin percentages can be translated directly into lignin abundances if we take their values as representing samples from a distribution of tissues with varying lignin contents. Because lignin abundance is not uniform within plant tissues, the variability in $\delta^{13}\text{C}$ between samples can be cast as variability in lignin content. We can interpret this variability in the context of a model of expected variance in the composition of tissues by rearranging equation (1) to quantify the expected percentage of lignin in each sample. In this case, if the original abundance of lignin in medullosan wood was similar to percentages found in angiosperms (mean, 22%; fig. 3) or conifers (mean, 29.8%; fig. 2), then we can express individual variability in $\delta^{13}\text{C}$ of samples as lignin percentages. Briefly, we can assume that the mean carbon isotope value of xylem ($\delta^{13}\text{C}_{\text{average_xylem}}$) reflects a lignin percentage that is similar to that of angiosperm wood

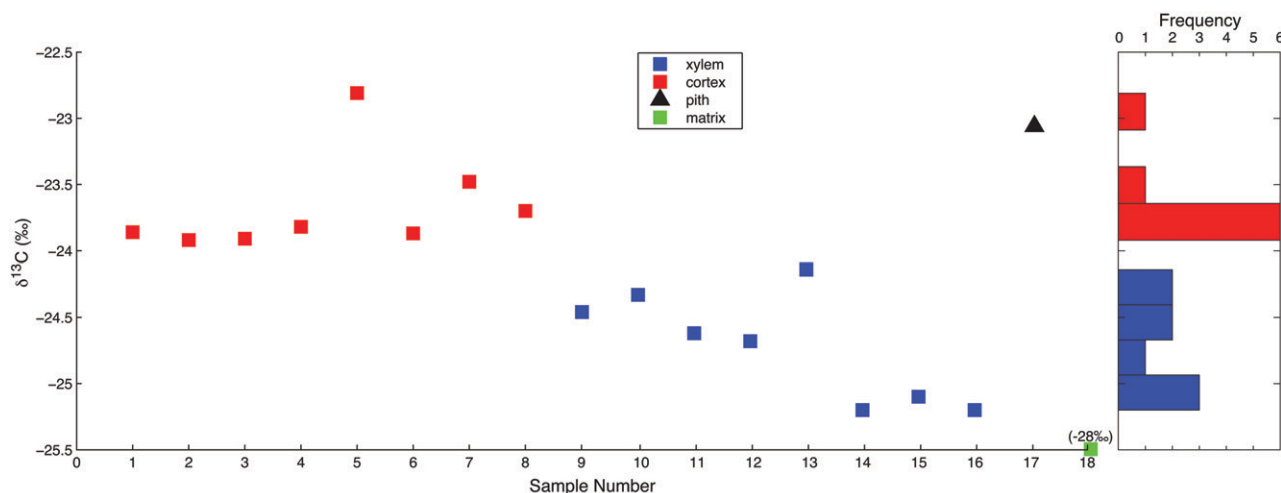


Fig. 6 Sample number and $\delta^{13}\text{C}$ values for *Medullosa anglica* var. *ioensis* stem. Medullosan xylem samples average $-24.72\text{‰} \pm 0.41\text{‰}$ and cortical samples average $-23.67\text{‰} \pm 0.38\text{‰}$. Cortical tissues are, on average, $1.04\text{‰} \pm 0.31\text{‰}$ heavier than xylem, implying a weak to unlignified cortex. The large difference from organic carbon in the nodule matrix provides evidence for spatially preserved, texturally specific information on the medullosan stem's tissues, rather than contamination from external sedimentary sources.

($X_{\text{angiosperm_wood}}$). If so, deviations from the mean xylem stable isotope composition by individual xylem samples ($\delta^{13}\text{C}_{\text{sample}}$) relative to the average difference between lignin and cellulose ($\Delta\delta^{13}\text{C}_{\text{cellulose-lignin}}$) will reflect differences in lignin percentages in each sample ($X_{\text{medullosan_wood}}$):

$$X_{\text{medullosan_wood}} = X_{\text{angiosperm_wood}} \times [1 - (\delta^{13}\text{C}_{\text{sample}} - \delta^{13}\text{C}_{\text{average_xylem}} / \Delta\delta^{13}\text{C}_{\text{cellulose-lignin}})]. \quad (2)$$

This, in turn, implies that differences in $\delta^{13}\text{C}$ between samples drawn from the cortex and the average $\delta^{13}\text{C}$ of the xylem reflect differences in lignin percentage and can be expressed as

$$X_{\text{medullosan_cortex}} = X_{\text{angiosperm_wood}} \times [1 - 100 \times (\delta^{13}\text{C}_{\text{sample}} - \delta^{13}\text{C}_{\text{average_xylem}} / \Delta\delta^{13}\text{C}_{\text{cellulose-lignin}})]. \quad (3)$$

Finally, if medullosan wood contained lignin abundances as high as those found in conifers, then the mean lignin percentage from conifer wood ($X_{\text{conifer_wood}}$, 29.8%; fig. 2) can be substituted into equations (2) and (3) to yield

$$X_{\text{medullosan_wood}} = X_{\text{conifer_wood}} \times [1 - (\delta^{13}\text{C}_{\text{sample}} - \delta^{13}\text{C}_{\text{average_xylem}} / \Delta\delta^{13}\text{C}_{\text{cellulose-lignin}})], \quad (4)$$

$$X_{\text{medullosan_cortex}} = X_{\text{conifer_wood}} \times [1 - 100 \times (\delta^{13}\text{C}_{\text{sample}} - \delta^{13}\text{C}_{\text{average_xylem}} / \Delta\delta^{13}\text{C}_{\text{cellulose-lignin}})]. \quad (5)$$

The results of this analysis are presented in figures 4, 5, 6, and 7. According to this method, medullosan cortical tissue has low abundances of lignin (maximally, 11%). This suggests that the medullosan cortex did not contain fibers or sclerids that were highly lignified, as would be expected if

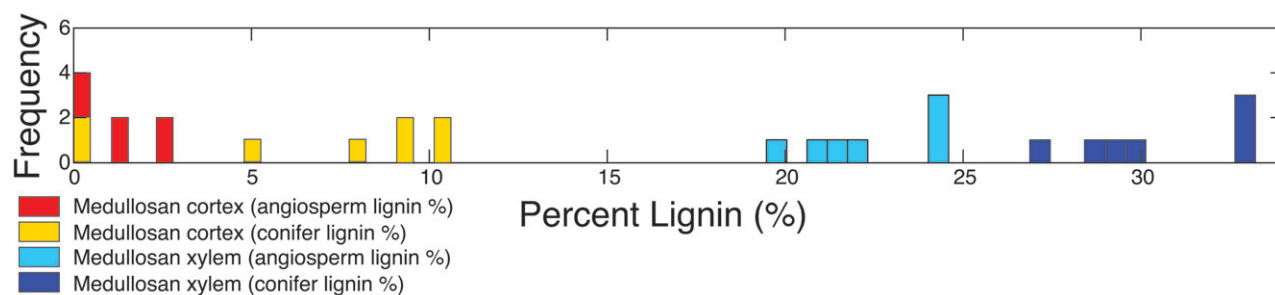


Fig. 7 Histogram of estimated lignin percentages within medullosan xylem and cortex tissues. If medullosan xylem originally contained lignin abundances similar to those of extant conifers (29.2%; table 1), cortical tissues contained maximally 11% lignin. If medullosan xylem contained lignin abundances similar to those of angiosperms (22%; table 1), cortical tissues contain little to no lignin. For comparisons with extant plants, see figure 3. Extremely positive $\delta^{13}\text{C}$ values ($> -20\text{‰}$, which yield negative lignin percentages in eqq. [3] and [5]) are assumed to reflect an absence of lignin and carbohydrate ^{13}C enrichment and are assigned a lignin abundance of 0%.

cortical tissues were integral to the structural support of the plant. Even if medullosan cortical tissues contained fiberlike cells for structural support, as angiosperms do, the small spatial scale of microdrilling measurements and the precision of $\delta^{13}\text{C}$ analyses would be sensitive to rare cells with $\sim 40\%$ lignin content; we do not find any evidence of such tissues in our analysis. Petioles have higher $\delta^{13}\text{C}$ values, which implies a richer carbohydrate component, consistent with their proximity to sites of photosynthesis. Most importantly, tissues expected to be rich in cellulose (e.g., pith, petioles) appear very similar in isotopic composition to cortical tissues (fig. 4).

Discussion

Previous work has observed that fossil plants commonly express a smaller isotopic difference between lignified and unlignified tissue than is expected from the empirical difference between lignin and cellulose (Boyce et al. 2003). Our analysis can cast a new light on these observations. First, there is a limit to cell wall lignin content: all cells begin as parenchyma cells, with cell walls being composed primarily of cellulose and hemicellulose (Esau 1977). By comparing leaf cells with lignin content data from fibers, we can show that the maximum expected difference is $\sim 2.5\%$. The full expected difference between pure lignin and pure cellulose observed in living plant biopolymers ($4.1\% \pm 0.6\%$) will never be fully expressed in plant fossils until an isotope analysis can be performed with high precision on the scale of individual molecules. This is in agreement with values reported from the earliest vascular plants (Boyce et al. 2003).

The offset captured in $\delta^{13}\text{C}$ values between cortex and xylem is interpreted as reflecting differential lignification, but alternative ways to interpret the data do exist. Because secondary xylem cells are produced over time, whereas cortical tissues are produced relatively early in plant development, it is possible that the difference was caused by environmental change. It is difficult to rule out this hypothesis entirely (because microdrilling spot sizes cover 10–15 xylem cells), but three lines of evidence suggest that this is unlikely. First, the earliest-developed secondary xylem cells have the most negative $\delta^{13}\text{C}$ values, in contrast to the more ^{13}C -enriched cortical tissues; if there was a secular change in $\delta^{13}\text{C}$ source values (e.g., water stress early in development leading to more positive $\delta^{13}\text{C}$ values for carbohydrate-incorporated cortical tissues), then we would expect values of the earliest secondary xylem to be most similar to values from the cortex, rather than maximally different (fig. 5). Second, there does not appear to be any spatial pattern within xylem cells reflecting clear secular change, though our time-series resolution is limited. Finally, cortical tissues from petioles appear to be isotopically indistinguishable from stem cortical tissues, despite differences in their relative timing of growth; this implies that the source of carbon for cortical cells is consistently ^{13}C enriched over time. It may be possible that ecophysiological change lies at the heart of what we see as variance between two tissue types, but these distributions are essentially nonoverlapping.

The presence of a spatially resolved, systematic 1.04% isotopic difference between xylem and cortex in a well-preserved specimen of *Medullosa anglica* reflects systematic differences in underlying lignin concentration. These results

demonstrate that the cortical tissues of the stem lacked sufficient lignin content to provide structural support, particularly given that medullosans have large leaf areas and lack accessory structures on stems, such as stilt roots or a primary thickening meristem, to provide buttressing strength (Delevoryas 1955; DeMason 1983; Mosbrugger 1990). In this light, it is instructive to contrast the anatomy of this medullosan with that of a typical arborescent gymnosperm or angiosperm (fig. 8). Virtually all gymnosperms and dicot angiosperms that are freestanding trees develop a solid cylinder of xylem from the vascular cambium that confers structural rigidity to the entire plant (Esau 1954; Niklas 1992). The vascular cambium of *Medullosa*, on the other hand, developed anastomosing segments of vascular tissue, rather than a cylinder, and the majority of the area of a medullosan stem was composed of cortical tissue or leaf bases, and not xylem (Delevoryas 1955; Basinger et al. 1974; Pfefferkorn et al. 1984; Wnuk and Pfefferkorn 1984; Mosbrugger 1990). The low abundances of lignin in cortical tissue suggest that the external portion of the plant would have been soft, rather than woody, leaving the small amount of xylem as the sole support of the entire plant.

Although our sample size is somewhat limited, it is possible to reflect on the identity of cortical tissues in this type of medullosan. Although many ferns possess sclerified tissue in the hypodermis of their petioles, the absence of lignin from these specimens suggests that medullosan fronds had a collenchymatous hypodermis, which is used to accommodate changes in loading in many extant angiosperm petioles (Niklas 1991). Collenchyma would contribute substantial flexibility to the medullosan stem and petioles; tissue-level analysis of the extant fern *Angiopteris*, which also contains fronds in excess of 4 m in length, could provide a useful perspective on this biomechanical problem.

Taken together, the large leaf area, absence of accessory support structures, and low lignification within this fossil's cortex suggest that medullosan fossils with this anatomical and biophysical configuration may not have been arborescent. This observation supports hypotheses from independent techniques, including hydraulics (Wilson et al. 2008; Wilson and Knoll 2010), morphology (Delevoryas 1955), anatomy (Pfefferkorn et al. 1984; Wnuk and Pfefferkorn 1984; Hamer and Rothwell 1988; Mosbrugger 1990; DiMichele et al. 2006), biomechanics (Rowe et al. 1993; Rowe and Speck 2004), and comparative biophysics (Masselter et al. 2006). Although some medullosans were certainly arborescent (fig. 1C), there are good reasons to hypothesize that the morphotype characterized in this study was not capable of supporting itself throughout its life history. Both ideas may well be correct for different members of this group, and the diversity of architectures within the genus *Medullosa* may reflect a broad range of plants aggregated within a single genus name (Pfefferkorn et al. 1984; Wnuk and Pfefferkorn 1984).

In some ways this conclusion should not be surprising: climbing architectures are common today in monocots, basal eudicots, and ferns. Furthermore, analysis of Young's modulus values of fossil plants and comparative biology have shown that this mode of growth was common in the Paleozoic era, along with other, more unusual growth forms rarely

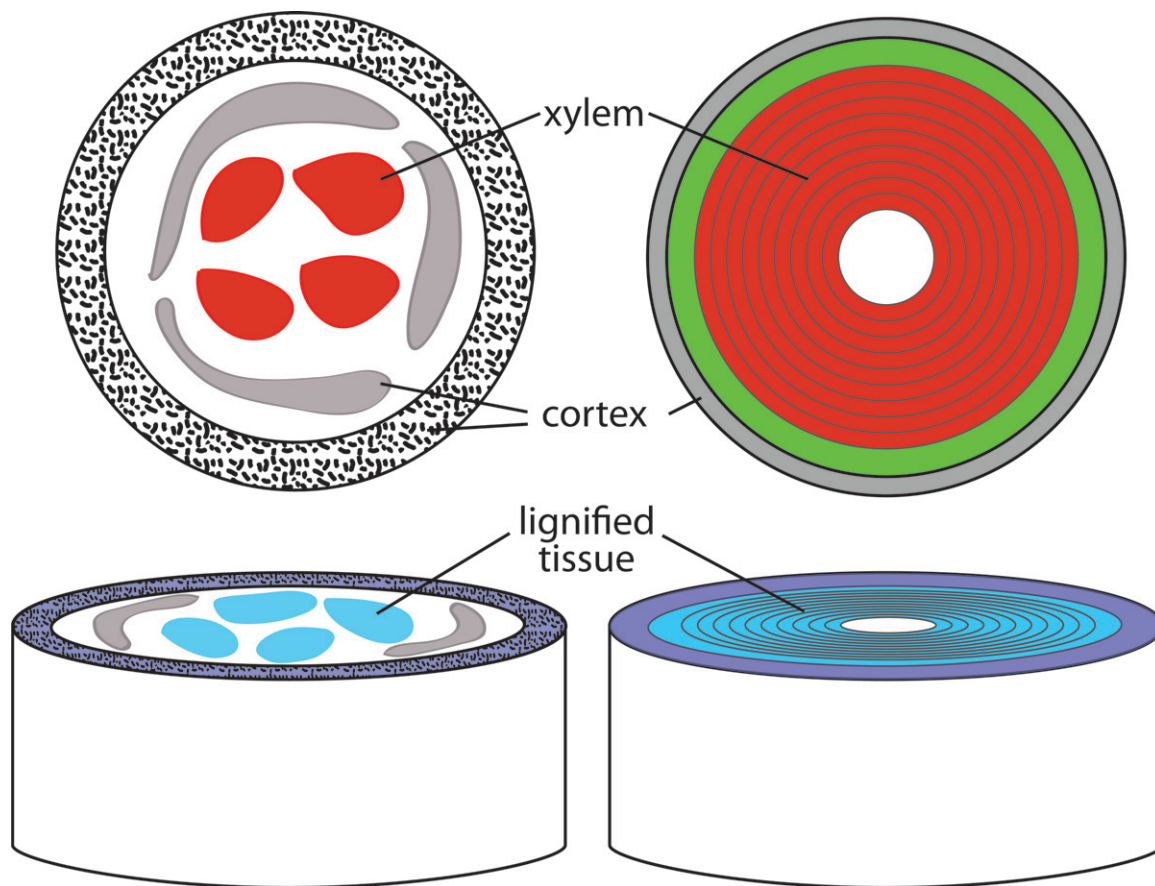


Fig. 8 A three-dimensional cross section of medullosan anatomy, juxtaposed with a schematic diagram of an arborescent plant (either angiosperm or gymnosperm). In arborescent plants that are entirely freestanding through their life cycle, substantial structural support is provided by the vascular cylinder: xylem walls for conifers and fibers in angiosperms. The medullosan architecture uncovered in our study does not fit this model; xylem contains more lignin than do the cortical tissues, but the cells are too wide to provide structural support for the plant (Wilson et al. 2008).

seen in extant plants (Rowe et al. 1993; Pfefferkorn et al. 2001; Rowe and Speck 2004; Masselter et al. 2006).

Taken as a whole, these results suggest that lignin abundances, and thereby growth habit and stature, can be resolved in well-preserved fossils. Because coal balls are a major component of the Carboniferous plant fossil record, the canopy architecture of coal swamp forests in North America and western Europe can be informed by using chemical analysis of isotope ratios in well-preserved fossil plants. If it is true that lax-stemmed morphologies are common among stem group seed plants, as is suggested for *Lyginopteris*, *Callistophyton*, and *Medullosa*, then this implies that the diversification of early seed plants may have occurred in an environment similar to that of the radiation of early angiosperms: the forest understory (Feild et al. 2004).

Conclusions

Our analysis shows the utility of carbon isotopes for revealing tissue-specific patterns in the structural biochemistry of fossil plants. In particular, the ability to differentiate tissues from xylem provides a way to ask questions about the degree

of lignification of additional tissue types. Results from stable isotope analyses of tissues within a specimen of the Late Paleozoic seed plant *Medullosa* reveal low lignin contents in cortical tissues, supporting hypotheses that the narrow-stemmed medullosan morphotypes had a climbing or a lax-stemmed morphology. Along with other climbing morphologies previously reported within the genus *Medullosa*, these results reflect the ecological diversity within stem group seed plants. Further geochemical work across the early seed plant clades will shed light on the unique biophysical strategies employed by plants in the coal swamps of the Carboniferous Period.

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